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Nucleotide and Amino Acid Sequence Variations in the L1 Open Reading Frame of Human Papillomavirus Type 6

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Human papillomavirus (HPV) type 6 induces benign tumors such as condyloma acuminata and laryngeal papilloma. The HPV-6 DNA has been thought to be a heterogeneous group of subtypes and variant-types. To examine sequence variations of the HPV-6 L1 ORF, we analyzed by single-strand conformation polymorphism (SSCP) and by DNA sequencing 21 specimens from condyloma acuminata and laryngeal papilloma that harbor HPV-6 DNA. PCR products of HPV-6 DNA were digested with 6 restriction enzymes yielding 8 fragments, which were then analyzed by SSCP. The resolution patterns showed that the L1 coding sequences were separated into three SSCP groups, I, II, and III, and two minor groups, (I) and (III). By sequencing the five representatives of each SSCP group and by comparing these sequences with those of HPV-6a [Hofmann et al., 1995] and HPV-6b [Schwarz et al., 1983], we identified base substitutions at 20 positions in the L1 coding region and an amino acid substitution in one case. ***J. Med. Virol. 53:19–24, 1997.***

KEY WORDS: condyloma acuminata; laryngeal papilloma; human papillomavirus; L1 ORF; sequence variation; variant type

INTRODUCTION

HPV-6 subtypes HPV-6a, -6b, and -6c were identified originally in condyloma acuminata [Gissmann et al., 1983], and subtype HPV-6d was identified in Buschke-Löwenstein [Boshart and zur Hausen, 1986], based on the DNA fragment sizes resulting from digestion with restriction enzyme *Pst*I. Subtypes of HPV-6 DNA from respiratory tract lesions have been separated further into HPV-6a to -6f, based on genomic DNA digests with combinations of restriction enzymes [Ward and Mounts, 1989]. Thus HPV-6 has been thought to be highly polymorphic.

However, sequence variations of the HPV-6 genome have been examined mainly in the noncoding region (NCR), which is the most variable region of the HPV genome, and in early genes E6 and E7 [Boshart and zur Hausen, 1986; Rando et al., 1986; Kasher and Roman, 1988; de Villiers et al., 1989; Kulke et al., 1989; Hrisomalos et al., 1990; Rübben et al., 1992; Roman and Brown 1995; Henizel, et al., 1995; Grassmann et al., 1996]. Rübben et al. [1992] reported 4 major subtypes by restriction enzyme cleavage analysis of the NCR, and Heinzel et al. [1995] reported 19 HPV-6 variants determining the nucleotide sequences of part of the NCR, although enhancer activities of these variants on the E6 promoters differed by only two- to threefold. Grassmann et al. [1996] reported a high degree of variability of amino acids within the E6 ORF in HPV-6 genomes associated with laryngeal papilloma and with condyloma of a pregnant woman, but not in that associated with vulval condyloma.

Recently, the complete nucleotide sequence of HPV-6a was determined by Hofmann et al. [1995] and was compared with the sequence of HPV-6b determined by Schwarz et al. [1983]. Nucleotide substitutions were found in all ORFs, and amino acid changes were found in all ORFs in the early-gene region and in the L2 ORF. However, no amino acid change was found in the L1 ORF. To date, the sequences of small parts of HV-6 L1 and L2 ORFs have been determined and nucleotide variations of these ORFs have been reported [Icenogle et al., 1991; Yaegashi et al., 1993].

Previously, we found deletions in the L1 ORF of HPV-6 from laryngeal papilloma [Suzuki et al., 1995]. In this paper, we examined sequence variations of the L1 ORF of 21 samples of HPV-6 DNA from condyloma and laryngeal papilloma by SSCP and sequencing of the entire L1 ORF, and found three major SSCP

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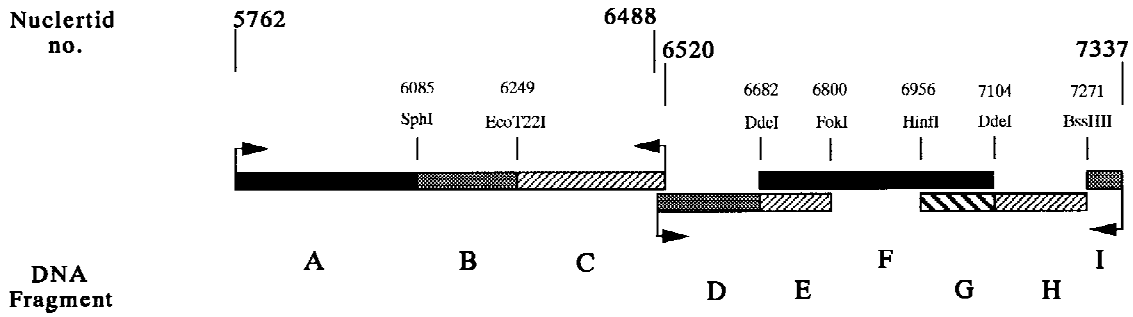

Fig. 1. Map of DNA fragments used for SSCP analysis. Arrows indicate PCR primers. The positions of the primers and the restriction sites are indicated by the nucleotide number of HPV-6a [Hofmann et al., 1995]. A to H show restriction fragments used for SSCP.

groups, I, II, and III, but found no amino acid change in L1 ORFs of HPV-6 from laryngeal papilloma.

MATERIALS AND METHODS

Biopsy Specimens and HPV DNAs

Biopsy specimens from 8 patients with recurrent laryngeal papillomas (KP-1 to KP-8) and 41 patients with condyloma acuminata (CA-35 to CA-75) (49 specimens total) were obtained from hospitals affiliated with the School of Medicine, Chiba University. The ages of these patients ranged from 2 to 53 years. Twenty-one specimens harbored HPV-6, and the remaining 28 specimens harbored HPV-11. In this study, 21 specimens which harbored HPV-6 were used for SSCP analysis.

Southern Hybridization and Polymerase Chain Reaction (PCR)

Three to 5 μg of total DNA extracted from each specimen were digested with the restriction endonuclease *Pst*I and analyzed by Southern blot hybridization as described previously [Tsuchiya et al., 1991]. The specimens that harbored HPV-6a or HPV-6c were used for sequence-variation analysis of the L1 ORFs. In some experiments, HPV-6b DNA cloned into pBR322 [Gissmann et al., 1983] was used as an internal marker. For PCR amplification, two primer sets with sequences corresponding to HPV-6a [Hofmann et al., 1995] were prepared. For the 5′ half of the L1 ORF, the primer set, L1-u1 (5′-CGTAAACGTATTCCCTTATTTTTT-3′)/L1-d1 (5′-AAAAATAATCTATCACCATA-3′), which corresponded to nucleotides (nt) 5762-5785 and nt 6501-6520, was used. For the 3′-half of the L1 ORF, a primer set, L1-u2 (5′GGCTGCAGACCCATATGGTG-3′)/L1-d2 (5′-AACAGTACATACACACATAT-3′), which corresponded to nt 6488-6507 and nt 7318-7337, was used (Fig. 1). Amplification was carried out in a 50 μl reaction mixture in 35 cycles under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2.5 min. The PCR products were cloned into the multicloning site of the phagemid pTV118/119N (Takara Shuzo Co. Ltd., Japan) or pBluescriptII SK(±) (Promega Co. Ltd., Madison, WI). The DNA sequence was then determined by the dideoxy method with Sequenase ver.2 (United States Biochemical, Cleveland, OH) using synthetic oligonucleotide primers that corresponded to the sequence of the L1 ORF of HPV-6a [Hofmann et al., 1995].

Single Strand Conformation Polymorphism (SSCP) and Sequencing

For SSCP analysis, PCR amplification was carried out according to the methods described by Ravnik-Glavac et al. [1994], with minor changes. The DNA samples used for Southern blot analysis were diluted 1:100, and 1 μl of the dilution was used for PCR. Of the 50 μl of the PCR products, 15 μl was digested with restriction enzymes in a 20 μl reaction mixture. The PCR products of the 5′-half of the L1 ORF were digested with a mixture of restriction enzymes *Sph*I and *Eco*T221. Three fragments, A, B, and C, were obtained (Fig. 1). The PCR products of the 3′-half were digested with mixtures of restriction enzymes *Dde*I and *Fok*I, *Dde*I and *Hinf*I, and *Dde*I and *Bss*HII. Six fragments, D, E, F, G, H, and I, were obtained. The E- and G-fragments overlapped with the F-fragment (Fig. 1). Two μl of each reaction mixture was mixed with 8 μl of DNA-sequencing stop buffer (Sequenase ver.2). The 10 μl mixture was heated at 90°C for 5 min and 1 μl of the solution was loaded onto a 6% polyacrylamide nondenaturing gel and electrophoresed at 4°C. To determine the sequence of the 3′-end 21 bp in the L1 ORF from nt 7272 to nt 7292, the PCR product was directly sequenced. Briefly, the HPV-6 DNAs were amplified by PCR using the primer set NCR1/NCR2 [Suzuki et al., 1995] and the PCR products were purified by agarose gel electrophoresis. One-twentieth of the purified product was then amplified asymmetrically in both directions with the same primers. Primers were removed by three successive centrifugations in a Centricon-30 column (Millipore Co., Bedford, MA). The asymmetrical PCR products were then sequenced in both directions with a Sequitherm cycle sequencing kit (Epicentre Technologies, Madison, WI) with a primer in a direc-

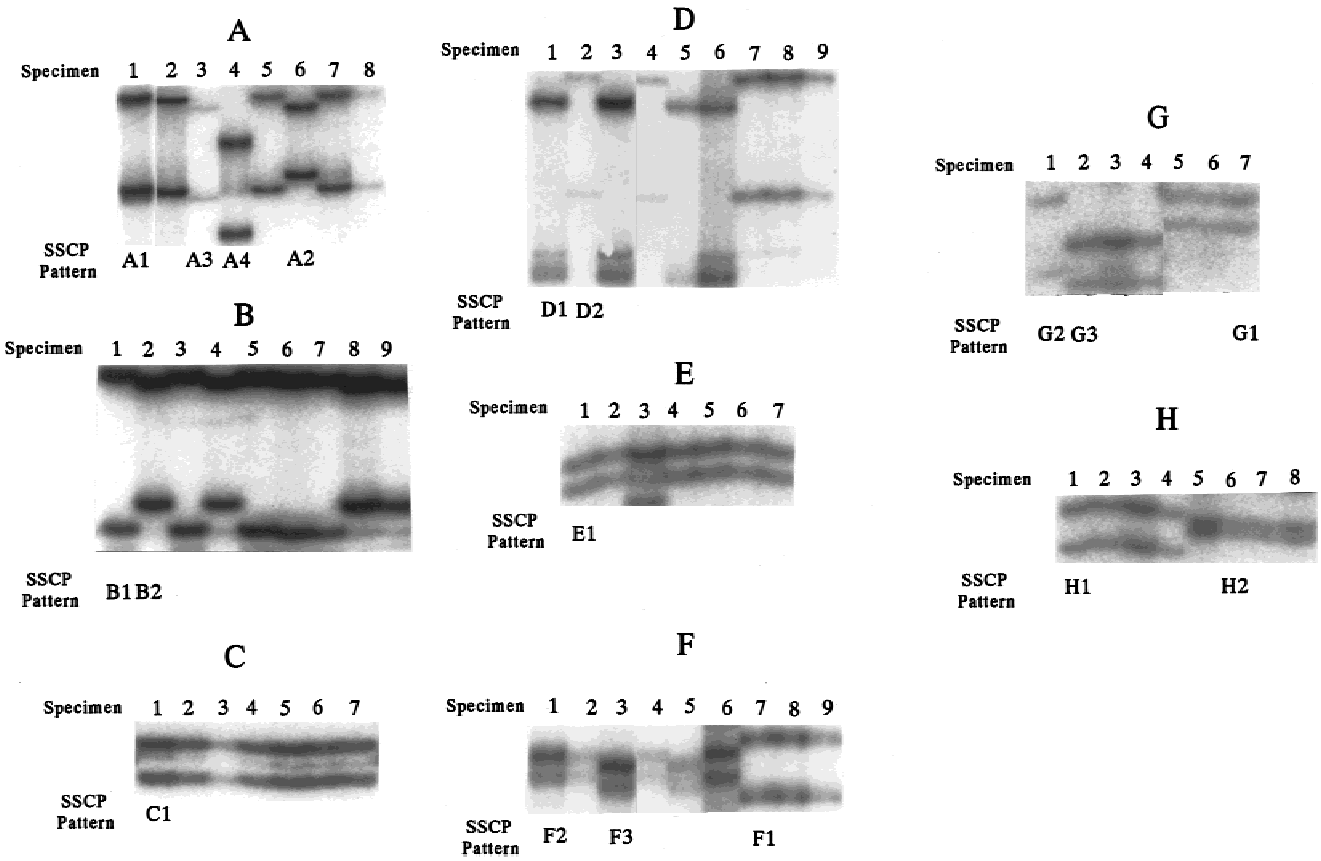

Fig. 2. SSCP patterns of **A- to H-**fragments (selected results). Resolution patterns of the A- to H-fragments A1-4, B1-2, C1, D1-2, E1, F1-3, G1-3, and H1-2 are shown. Specimens of A- to H-fragments: A-fragment, 1 to 8; KP5, CA-38, CA-43, CA-45, C-49, C-44, C-60, C-38. B-fragment, 1 to 9: C-43, C-45, C-49, C-54, C-60, C-67, C-69, C-35, C-42. C-fragment, 1 to 7: C-65, C-62, C-51, C-50, C-47, C-38, C-44. D-fragment, 1 to 9: KP2, KP3, KP5, C-45, C-38, HPV-6b, C-35, C-42, C-52. E-fragment, 1 to 7: C-42, C-35, HPV-6b, C-67, C-60, C-54, C-49. F-fragment, 1 to 9; KP2, KP3, KP5, C-45, C-38, HPV-6b, C-35, C-42, C-52. G-fragment, 1 to 7: C-54, C-35, C-42, C-52, C-49, C-60, C-67. H-fragment, 1 to 8: C-54, C-35, C-42, C-52, HPV-6b, C-49, C-60, C-67.

tion reverse to that of the initial asymmetrical amplification.

RESULTS

Subtype Analysis of HPV-6

Total DNA from laryngeal papilloma and condyloma acuminata were digested with *Pst*I and analyzed by Southern blot hybridization. The HPV-6 DNAs in these specimens were classified into HPV-6a and -6c as reported by Gissmann et al. [1983]. Two samples of HPV-6a DNA, KP2 and KP4, from laryngeal papilloma had large deletions in the L1 ORFs as reported [Suzuki et al., 1995]. Eighteen HPV-6a samples and 3 HPV-6c samples were used to analyze sequence heterogeneity.

SSCP Analysis

The L1 ORFs of HPV-6a and HPV-6c genomes were amplified by PCR using two sets of primers. PCR products were then digested with different combinations of restriction enzymes (see Materials and Methods). From the PCR products of the 5′-half of the L1 ORF, A-, B-, and C-fragments were produced, and from the PCR products of the 3′-half, D-, E-, F-, G-, H-, and I-fragments were produced. The resolution patterns of the A-, B-, and C-fragments were analyzed on the same gel, and the resolution patterns of the D- and F-, E-, G-, and H-fragments were analyzed separately after digestion with different combinations of restriction enzymes. The I-fragment was too small to analyze by SSCP. The A-fragment, derived from 21 HPV-6 samples, showed 4 different resolution patterns (parts of the results are shown in Fig. 2-A), the B-, D-, F-, G-, and H-fragments showed 2-, 2-, 3-, 3-, and 2-different resolution patterns, respectively, and the C- and E-fragments showed only one pattern (Fig. 2B–H). These results suggested that base substitution(s) occur in all fragments except C and E, and also that the HPV-6 L1 ORFs from 21 specimens were composed of three major SSCP groups, I, II, and III, and two minor groups, (I) and (III) (Table I). The resolution patterns of the A- to C-fragments of KP-2 and the pattern of the D- to H-fragments of KP-4 could not be determined because KP-2 had a large deletion in the 5′-half of the L1 ORF and KP-4 had one in the 3′-half [Suzuki et al., 1996].

To examine a correlation between these SSCP groups and subtypes reported by Rübben et al., (1992), the

TABLE I. Variations of HPV-6 L1 ORF by SSCP Analysis

SSCP group	SSCP pattern	Specimen	Subtype of HPV-6
I	A2 B2 C1 D1 E1 F2 G1 H1	KP-3, C-44, C-51, C-54	HPV-6a
(I)	A4 B2 C1 D1 E1 F2 G1 H1	C-45	HPV-6a
II	A2 B2 C1 D1 E1 F1 G3 H1	C-35, C-42, C-52	HPV-6c
III	A1 B1 C1 D2 E1 F3 G2 H2	(KP-4)*, KP-5, C38, C-47, C-49, C-50, C-60, C-62, C-65, C-67, C-69	HPV-6a
(III)	A3 B1 C1 D2 E1 F3 G2 H2	(KP-2)*, C-43	HPV-6a

*The HPV-6a L1 ORF in KP-2 and KP-4 samples contains a large deletion [Suzuki et al., 1995].

NCR regions of these HPV-6 genomes were amplified by PCR, digested with restriction enzymes, and separated by agarose gel electrophoresis, as described by Rübben et al., (1992). It was found that the restriction patterns of the NCRs of the SSCP groups, I(I), II, and III(III), were identical with those of subtypes ACVI, ACVIII, and ACVII, respectively (data not shown).

Sequence Variations

To determine the nucleotide sequences of the L1 ORFs, the PCR products of KP-2, KP-3, KP-5, C-42, C-43, and C-45 were cloned into plasmids and the sequences were determined. A portion (21 bp) of the L1 ORF 3′-end of the other HPV-6 samples was determined by direct sequencing of the PCR products. As shown in Figure 3, base substitutions were observed at no more than 11-nucleotide positions in all L1 ORFs examined, when those sequences were compared with that of HPV-6a [Hofmann et al., 1995] and HPV-6b [Schwarz et al., 1983]. The amino acid substitution (Asn to Thr) was found in only in C-45 at amino acid 55 of the L1 ORF. All these base substitutions were readily explained by differences in the resolution patterns of the A-, B-, D-, F-, G-, and H-fragments on SSCP gels (Fig. 2).

Homology of the Nucleotide Sequences

As shown in Figure 3, the base substitutions in the L1 ORFs of SSCP group-I (KP-3), SSCP group-II (C-42), HPV-6b, and SSCP group-III (KP-5) were found at 6, 9, 8, and 10 positions, respectively, when these sequences were compared with that of HPV-6a [Hofmann et al., 1995]. A region from nt 5993 to 6218 was most variable, as base substitutions occurred at 4 positions. The SSCP group-I (KP-3) closely resembled HPV-6a; a region from nt 6626 to 7262 was identical. However, the SSCP group-III (KP-5) more closely resembled HPV-6b than it resembled HPV-6a. The SSCP group-II (C-42), which has been classified into subtype HPV-6c, closely resembled the SSCP group-I (KP-3), and the differences were found only at 3 positions, at nt 6830, 7076, and 7078.

DISCUSSION

The SSCP method for mutation detection has been shown to be 75–98% sensitive [Ravnik-Glavac et al., 1994]. Thus it is advantageous to apply SSCP analysis to determine the sequence heterogeneity of HPV-6 DNA within many specimens. Using SSCP, our results were consistent with those using sequencing. For example, the resolution-pattern differences between A1 and A3 resulted from a single base substitution in the 299 bp long A-fragment. The differences between D1 and D2, F1 and F2, A1 and A2, A2 and A3, and F1 and F3 were resulted from 2-, 3-, 4-, 5-, and 6-base substitutions, respectively.

Several groups have demonstrated that HPV-6a is the predominant subtype found in condyloma acuminata biopsies from patients in the United States of America and in Europe [Gissmann et al., 1983; Brown et al., 1993]. A recent report suggested that HPV-6a is the HPV-6 prototype [Kitasato et al., 1994]. In the present experiment, HPV-6a was the major subtype of HPV-6 in the specimens from laryngeal papilloma and condyloma acuminata, whereas no HPV-6b was found. Rübben et al. [1992] analyzed heterogeneity of the HPV-6 NCR by restriction enzyme digestion and reported that 41 of 42 HPV-6 isolates were separated into four subtypes, ACV1 to ACV4. By SSCP and sequencing analyses it was found that 18 of 21 HPV-6 isolates were separated into SSCP groups, I, II, and III, which have NCRs corresponding to ACVI, ACVIII, and ACVII, respectively (data not shown). Therefore, SSCP groups, I, II, and III would be major subtypes of HPV-6 that are prevalent, at least, in Japan and Europe.

At present, at least 19 HPV-6 variant NCRs have been reported [Heinzel et al., 1995; Roman and Brown, 1995; Grassmann et al., 1996]. However, no significant difference on the enhancer activity of the NCR [Heinzel et al., 1995; Grassmann et al., 1996] and no strong correlation between these variants and lesion location, sex of patient, or pregnancy status has been reported [Roman and Brown, 1995]. In contrast, high amino acid sequence variability within E6 ORF was found in HPV-6 associated with laryngeal papilloma and with vulva condyloma from a pregnant woman [Grassmann et al., 1996].

The complete DNA sequence of HPV-6a has been determined [Hofmann et al., 1995], and has been shown to have over 97% identity to the sequence of HPV-6b. Base substitutions in the L1 ORF were found at 8 positions, though all resulted in silent mutations (with no amino acid change). Yaegashi et al. [1993] described an amino acid change in the L2 ORF of HPV-6a compared with HPV-6b, and the same change has been confirmed by Hofmann et al. [1995]. From these results it has been suggested that the L1 ORF is the most conserved ORF of HPV-6 in terms of the amino acid sequence.

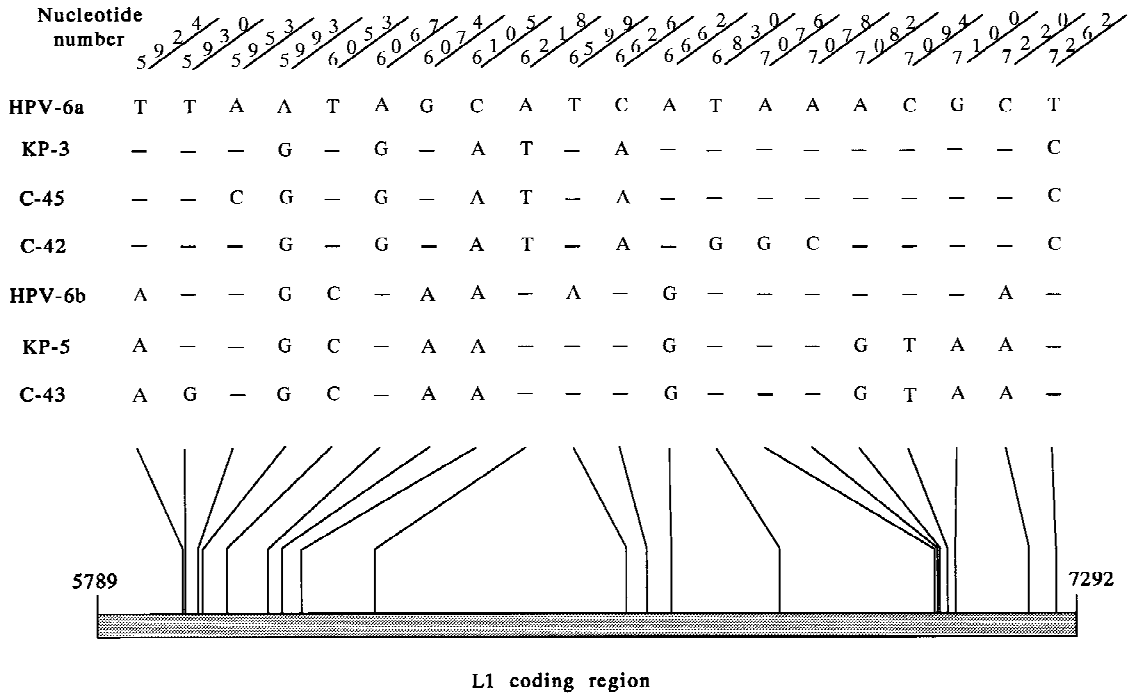

Fig. 3. Nucleotide substitutions in the L1 ORFs. The nucleotide numbers correspond to HPV-6a [Hofmann et al., 1995] and indicate positions in which nucleotide substitutions were found. Dashes (-) indicate the nucleotide identity with HPV-6a.

Here we found a single amino acid change, Asn to Thr, at amino acid number 55 in C-45; this is the first example of an amino acid change in the HPV-6 L1 ORF. The sequence of the C-45 L1 ORF had only one base substitution at nt 5953, which resulted in the amino acid change, when it was compared with other SSCP group-I sequences, such as KP-3. Therefore it is necessary to examine more HPV-6 samples to conclude whether or not this mutation is specific for C-45.

Heterogeneity of the nucleotide sequences of the L1 ORFs in HPV-6 DNAs isolated from geographically separated locations was found by PCR amplification, sequencing from nt 6508 to nt 6662, corresponding to the nucleotide numbers of HPV-6a, and by comparison of the sequence with that of HPV-6b [Icenogle et al., 1991]. They reported base substitutions at nt 6599, 6626, and 6662 in HPV-6 from the states of Georgia and Alaska in the United States, from India, and from the Philippines. We found that the SSCP groups III and (III), which included KP-5 and C-43, had the same substitutions as did Georgia-B5, India-D4, and Philippines-A4 at nt 6599, and that the SSCP groups I and (I), which included KP-3 and C-45, had the same substitutions as did Georgia-B1 and Philippines-A6 at nt 6599, 6626, and 6662. In our work, the SSCP groups III and (III) comprised 13 of the 21 samples (62%). Thus the most prevalent HPV-6 in Japan might also be the variant type prevalent in the state of Georgia, in India, and in the Philippines. The L1 ORF of HPV-6a, sequenced by Hofmann et al. [1995], has the same substitutions as those of Alaska-C36 and Georgia-B1 at nt 6599 and 6662. However, no HPV-6 L1 ORF in our samples showed identical base substitutions with Alaska-C36 and Georgia-B4 at nt 6599, 6662, or Georgia-G4 and -G7 at nt 6599, 6626.

Previously we have shown a large deletion in the L1 ORFs of HPV-6a from laryngeal papilloma [Suzuki et al., 1995]. These truncated L1 proteins would not assemble into virus particles, and would not induce conformation-dependent neutralizing antibody. However, we did not find amino acid substitutions in the L1 ORFs of HPV-6 associated with laryngeal papilloma. Therefore, deletions or amino acid changes in the L1 ORF, should they occur, might not be a major cause of rapid growth of the tumor or of its recurrence. It would be interesting to examine a possible correlation between rapid replication of the HPV-6 genome in laryngeal papilloma and specific amino acid change(s) of oncogenic protein(s) E6 and/or E7.

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